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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : C12Q 1/00, 1/68, 1/70, C12N 13/00, 9/00, G01N 33/573	**A1**	(11) International Publication Number: **WO 00/17386** (43) International Publication Date: 30 March 2000 (30.03.00)

(21) International Application Number: PCT/US99/21863

(22) International Filing Date: 21 September 1999 (21.09.99)

(30) Priority Data:
60/101,172   21 September 1998 (21.09.98)   US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ASSAYS AND NOVEL CELLULAR TARGETS FOR THERAPEUTIC AGENTS TO TREAT RETROVIRAL INFECTION

(57) Abstract

Assays and test kits for identifying compounds that can interfere with early steps of the retroviral life cycle in a host cell are provided. Assays and test kits for identifying novel cellular enzymes needed to complete integration of retroviral DNA into cellular DNA are also provided. These assays are based on the discovery that cellular enzymes are needed to complete the repair steps of retroviral integration, and lack of such enzyme(s) causes cell death upon infection with a retrovirus. One required enzyme is a DNA-dependent protein kinase (DNA-PK). Methods for interfering with the retroviral life cycle in an infected host cell are also provided, which involve inhibiting DNA-PK or any other enzyme needed to complete the repair step of retroviral integration.

ASSAYS AND NOVEL CELLULAR TARGETS FOR
THERAPEUTIC AGENTS TO TREAT RETROVIRAL INFECTION

This application claims priority to U.S. Provisional Application No. 60/101,172, filed September 21, 1998, the entirety of which is incorporated by reference herein.

5   This invention was made with United States government support awarded by the NIH-NCI, Grant No. CA71515. The United States government has certain rights in this invention.

10 FIELD OF THE INVENTION

This invention relates to the field of retroviruses and pathological conditions caused by retroviruses. In particular, this invention provides new assays to screen for anti-retroviral drugs, and a novel
15 cellular target for drug development.

BACKGROUND OF THE INVENTION

Several scientific publications are referenced in this patent application to describe the state of the
20 art to which the invention pertains. Each of these references is incorporated by reference herein, in its entirety.

Retroviruses cause several diseases and pathological conditions, including a variety of tumors
25 and leukemias. For instance, human inmmunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) in humans. Another significant disorder, adult T-cell leukemia-lymphoma, is

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caused by the retrovirus HTLV I (human t-cell leukemia
virus type I). HTLV I also has been associated with
other diseases, such as tropical spastic paraparesis and
HTLV I-associated myelopathy. Moreover, many animal
5   diseases of agricultural and veterinary importance are
known to be caused by retroviruses. These include avian
sarcoma leukosis virus (ASLV), feline leukemia virus
(FeLV), bovine immunodeficiency virus (BIV) and equine
infectious anemia virus (EIAV), among others.

10   The development of antiviral agents to reduce
or eliminate retrovirus-caused diseases has been a focus
of intensive research over the past several years. One
major difficulty in this effort has been the propensity
of the viruses to mutate in response to exposure to a
15 particular antiviral agent and for resistant mutants to
be selected. It is for this reason that "cocktails"
comprising two or more antiviral agents often provide
more effective treatment for retroviral infection than do
single agents. However, over time, even a cocktail can
20 become ineffective due to the virus's ability to develop
resistance to the antiviral agents.

        Retroviruses encode several enzymes that are
assembled into the virus particle and whose activities
catalyze essential steps in the infectious cycle: (a)
25 protease (PR), (b) the polymerase and (c) ribonuclease H
(RNAse H) activities of reverse transcriptase (RT), and
(d) integrase (IN). Following attachment and penetration
of the retrovirus into the host cell, the RT activities
catalyze reverse transcription of the viral RNA into DNA,
30 and IN catalyzes the integration of the retroviral DNA
into the host cell DNA. The cellular transcription and
translation machinery is used to produce viral RNA from
the integrated viral DNA and thereafter to produce the
viral proteins.

35

Retroviral integration proceeds in three
distinct steps, the first two of which are catalyzed by
IN, and require specific sequences at the ends of the
viral DNA, including a conserved CA dinucleotide. In the
5   first step, referred to as "processing," two nucleotides
(typically) are removed from the 3' ends of the viral
DNA, immediately adjacent to the CA dinucleotide. In the
second step, referred to as "joining," these new 3'-OH
ends are joined to staggered phosphorus atoms in the host
10   DNA. The resultant molecule comprises the viral DNA
inserted into the host DNA with gaps on both strands due
to the staggered nature of the joining reaction. Hence,
the third step of integration, referred to as "repair,"
comprises filling in the gaps in host DNA produced by the
15   staggered insertion (catalyzed by a polymerase activity),
removing the non-complementary overhangs at the 5' ends
of the viral DNA (catalyzed by a nuclease activity), and
completion of the covalent closure (catalyzed by a ligase
activity).
20   In *in vitro* studies, IN has been shown to
catalyze the processing and joining steps of integration,
but not the repair steps. It has been surmised that
cellular enzymes may be required (either with or without
the participation of IN) for these steps; however, the
25   identity of the enzyme(s) involved has not been
elucidated. It would be a significant advance in the art
of medicinal biology to identify this enzyme or
combination of enzymes, for the purpose of further
elucidating the retroviral lifecyle and thereby providing
30   new information for developing antiviral drugs. In
addition, such enzymes themselves would be of particular
utility as targets for drug development, inasmuch as the
propensity of cellular enzymes to mutate in response to
an external stimulus is many-fold reduced as compared
35   with the propensity of the virus to mutate.

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SUMMARY OF THE INVENTION

According to one aspect of the present invention, a method of identifying cellular enzymes needed to complete integration of retroviral DNA into a

5   genome is provided. The method comprises: (a) providing a pair of retroviral vectors, one of the pair encoding a functional integrase and the other of the pair encoding a non-functional integrase; (b) providing samples of a cell line suspected of lacking at least one enzyme needed to

10   complete integration of the retroviral vector into the genome of the cell line; (c) infecting one of the samples with the retroviral vector encoding the functional integrase and another of the samples with the retroviral vector encoding the non-functional integrase; and (d)

15   measuring cell death in each of the samples, a greater amount of cell death in the sample infected with the retroviral vector encoding the functional integrase, as compared with the sample infected with the retroviral vector encoding the non-functional integrase, being

20   indicative that the enzyme lacking in the cell line is needed to complete integration of the retroviral DNA into the cell's genome. Test kits for carrying out the method are also provided.

According to another aspect of the invention, a

25   method is provided for identifying an agent that interferes with a retroviral life cycle at a step preceding completion of integration of retroviral DNA into a host cell genome. The method comprises: (a) providing a cell line that is unable to perform a DNA

30   repair step in integration of retroviral DNA into the cell's genome; (b) infecting with a retrovirus one sample of the cell line in the presence of a test compound suspected of interfering with the retroviral life cycle; (c) infecting with a retrovirus an equivalent sample of

35   the cell line in the absence of the test compound; and

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(d) measuring cell death of the infected samples of cell
lines, a decrease in amount of cell death in the presence
of the test compound as compared with in the absence of
the test compound being indicative that the test compound

5   is an agent that interferes with the retroviral life
cycle at a step preceding completion of retroviral DNA
integration into the cell's genome. In a preferred
embodiment, the cell line comprises a non-functional DNA-
dependent protein kinase. Most preferably, the cell line

10   is derived from a *scid* mouse.
A test kit for carrying out this method is also provided.
       According to another aspect of the present
invention, a method is provided for interfering with
retroviral replication in a host cell infected with the

15   virus. The method comprises inhibiting an enzyme of the
host cell needed to perform the repair step of retroviral
DNA integration into the host cell genome. Preferably,
the enzyme is a DNA-dependent protein kinase. In a
preferred embodiment, the inhibiting is accomplished by

20   exposing the cell to an exogenous agent that inhibits the
enzyme.
       Other features and advantages of the present
invention will be understood by reference to the drawings
and descriptions thereof, detailed description and

25   examples which follow.

BRIEF DESCRIPTION OF THE DRAWINGS

       Figure 1. Set of histograms showing that
retroviral infection is lethal to DNA-PK$_{cs}$ deficient, *scid*

30   pre-B cells.
       Figure 2. Schematic diagram of a retroviral
DNA construct containing an expressible *neo* reporter
gene, RCASPB-M(neo).
       Figure 3. Set of histograms showing that

35   killing of *scid* cells by a retroviral vector requires an

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active integrase. Below the histograms are shown two
retroviral constructs. The upper construct encodes an
active.integrase, whereas the lower construct contains a
mutation that produces a single amino acid substitution
5   (D64E) in the active site that renders the integrase
inactive.

Figure 4. Histogram showing that retroviral
infection induces the apoptotic pathway in *scid* cells,
and flow chart showing the basic steps in the apoptosis
10   pathway.

Figure 5. Histograms showing activation of
DNA-PK$_{cs}$ in human (HeLa) cell nuclear extracts. Cells
were exposed to different treatments, then lysed. The
p53 peptide serves as a substrate for phosphorylation by
15   DNA-PK. The histogram on the left shows the result of
treating cells with sheared DNA to activate the lysate.
The histogram on the right shows the results of infecting
or mock-infecting cells with a retroviral vector. No
additional exogenous DNA was added.

20   Figure 6. Graphs and histogram showing that
DNA-PK$_{cs}$ deficient *scid* cells are killed by HIV-based
vector infection. The graphs on the left show percent
viability over time. The histogram on the right shows β-
galactosidase activity in vector-infected cultures.

25   Figure 7. Histogram showing that AZT protects
scid cells from virus-mediated cell killing.

Figure 8. Histogram showing the effect of
Wortmannin on retroviral integration of HeLa cells (G418
resistant colonies).

30   Figure 9. Graph showing the effect of
Wortmannin on growth of HeLa cells.

Figure 10. Graph showing the effect of
Wortmannin on DNA-PK-related activity.

Figure 11. Graph showing the effect of
35   Wortmannin on viability of S33 cells. *Scid* cells (S33

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line) were treated with 0-2 μM Wortmannin and
simultaneously infected with the RCASPB-M virus as
described in Example 1.

5   **DETAILED DESCRIPTION OF THE INVENTION**

                In accordance with the present invention, it
        has now been discovered that the final step in retroviral
        DNA integration (the "repair" step) requires components
        of the cellular DNA double strand break (DSB) repair
10   pathway. Specifically, DNA-dependent protein kinase
        (DNA-PK) activity is necessary to complete the
        integration of viral DNA into the host genome.

                DNA-PK is composed of a DNA binding component
        (Ku) and a large catalytic subunit (DNA-PK$_{cs}$). DNA-PK
15   functions in DNA double strand break (DSB) repair and
        V(D)J recombination involved in generation of antibody
        diversity (see Lees-Miller, Biochem. Cell Biol. $\underline{74}$: 503-
        512, 1996). It is now known that the severe combined
        immunodeficient (*scid*) mouse, and cell lines derived
20   therefrom, produce a defective DNA-PK comprising a
        nonsense mutation in the carboxyl terminal region of the
        catalytic subunit (Blunt et al., Proc. Natl. Acad. Sci.
        USA $\underline{93}$: 10285-10290, 1996). *Scid* mice fail to develop
        mature T and B lymphocytes because their defective DNA-PK
25   is unable to carry out the functional rearrangement of
        the elements encoding the immunoglobulin and T-cell
        receptor genes (i.e., V(D)J recombination). *Scid* mice
        also are exquisitely sensitive to ionizing radiation,
        which is known to cause double strand DNA breaks. This
30   sensitivity also is believed to arise from their lack of
        a functional DNA-PK to perform the DSB repair.

                The present inventors infected mouse cell lines
        derived from *scid* or normal mice with one of three
        different retroviral vectors, derived from ASLV, MoMLV
35   and HIV, respectively. Infection with these viruses

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induced cell death in *scid*, but not normal cells. Cell
death was not inducible by heat-inactivated virus. To
determine whether the observed cell death was due to
retroviral integration, cells were infected with one of a

5   pair of avian retroviral vectors, RCA8KSneo and
RCAD64Eneo. Sequences of these viral DNAs are identical,
except RCAD64Eneo carries a mutation that encodes an
amino acid substitution that inactivates only the
integrase protein. RCA8KSneo was found to induce cell

10   death in *scid* cells, whereas the integrase-defective
RCAD64Eneo did not. These results, along with additional
experimental results set forth in the examples,
demonstrate that (1) cell death in *scid* lines is due to
retroviral integration; (2) cell death occurs via an

15   apoptosis pathway; and (3) DNA-PK$_{cs}$ is needed for
retroviral integration, at the "repair" step, which IN
fails to catalyze *in vitro*. It is possible that other
cellular enzymes are involved in the repair step of viral
integration, and that IN participates in this process;

20   however, the foregoing results demonstrate the necessity
of a functional DNA-PK to complete the integration.

In addition to the inventors' discovery of the
requirement for DNA-PK to complete retroviral
integration, the inventors have further discovered that a

25   certain percentage of a cell population may catalyze the
repair step of viral integration by an alternative
mechanism, which involves the ATM (Ataxia-telangiectasia
mutated) kinase. This discovery is described in greater
detail in Example 7. Accordingly, the ATM kinase may

30   serve as a secondary target for antiviral drug
development, as discussed in greater detail below.

The above-summarized discoveries made in
accordance with the present invention enable advances in
anti-retroviral drug discovery in two significant areas:

35   (1) rapid and convenient assays are now possible to

screen for drugs that inhibit early steps in the
retrovirus lifecycle, i.e., steps up to and including the
processing and joining steps catalyzed by IN; and (2) a
cellular enzyme (DNA-PK) involved in the retroviral life
5   cycle is now available as a target for drug development.

The present invention includes several types of
assays. For instance, as mentioned above, other cellular
enzymes besides DNA-PK may be needed or involved in the
repair step of retroviral integration, but regardless of
10   the number of enzymes involved in the repair step,
failure of a cell to carry out this step results in cell
death. Accordingly, in one type of assay of the present
invention, paired retroviral vectors, such as RCA8KSneo
and RCAD64Eneo, in which one member of the pair is
15   integrase-defective, can be used in combination with
knock-out or mutant cell lines for the rapid
identification of such cellular genes involved in
retroviral integration. The integrase-functional
retroviral vector will induce cell death in cells
20   deficient for a gene needed for retroviral integration.
If the gene is truly required for integration, infection
with the integrase-defective retroviral vector will have
no effect on the test cell line.

Due to the genetic similarity among retroviral
25   genomes, paired vectors such as RCA8KSneo and RCAD64Eneo
(which are ASLV derivatives) can be constructed for any
retrovirus. Retroviruses suitable for such modification
include, but are not limited to, HIV, SIV, MoMLV, HTLV,
FeLV, BIV and EIAV, among others.

30   In another type of assay, a *scid* cell line, or
any other cell line carrying a defect that results in
failure to carry out the repair step of retroviral
integration, is used in a rapid screen for agents capable
of impeding or inhibiting steps in the retroviral life
35   cycle, up to and including the processing and joining

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steps of integration catalyzed by IN. This includes
blocking the function of either viral or cellular genes
required during the early stages of viral propagation.

        In a preferred embodiment, *scid* cells are used
5   in assays of this type. However, other DNA-PK defective
cell lines also may be used (such as the rodent mutant
cell line, *xrs-5*, described by Lees-Miller et al.,
Science 267: 1183-1185, 1995), as may be any cell line
carrying a defect that causes failure to perform the
10   repair step of retroviral integration and thus trigger
apoptosis. Indeed, useful cell lines of this type can be
identified using the paired retrovirus assay described in
the preceding paragraphs.

        In the simplest form of this screening assay,
15   an integration repair-defective cell line, such as a *scid*
line, is infected with a replication-competent retroviral
vector in the presence or absence of a test compound
suspected of blocking early steps in the retrovirus
lifecycle. If the test compound indeed does impede or
20   block one or more of these early events, the cells will
continue to live. If the compound is ineffective, the
cells will die. This assay offers the distinct advantage
of a positive selection - the cells live if the candidate
drug is effective, but die if it is not.

25   To demonstrate the efficacy of this assay, *scid*
cells and normal cells were infected with virus in the
presence or absence of AZT, a known inhibitor of reverse
transcription. Inhibition of any step prior to
integration is predicted to reverse killing of *scid* cells
30   by the retrovirus. The results shown in Figure 7
demonstrate that AZT abrogates the cell-killing effect of
retrovirus infection, as expected.

        The screening assay can be refined through the
use of appropriate controls. For instance, assays
35   preferably are constructed so that the integration

repair-defective cell lines are compared with equivalent
control cell lines that are competent in integration
repair. In a preferred embodiment, *scid* cells and a
near-isogeneic normal cell line are used. Another useful
5 control is side-by-side infection with a paired set of
retroviral vectors as described above.

In any of the foregoing assays, cell death can
be measured in a variety of ways. One way is simply to
measure cell death, e.g., by trypan blue staining.
10 Another way is to measure expression of a selectable
marker gene or a reporter gene, the expression of which
is dependent on stable integration of the viral vector.
As defects in the integration repair step appear to cause
cell death via the apoptosis pathway, a preferred method
15 comprises measuring an indicator of apoptosis. A
particularly preferred embodiment utilizes measurement of
caspase activation, which is an early event in apoptosis.

The present invention also identifies DNA-PK as
a novel gene target for inhibition of retroviral
20 replication. Of particular interest in this regard is
the inhibition of HIV replication, as a new therapy for
AIDS. Current anti-HIV therapies target HIV proteins,
predominantly HIV reverse transcriptase and protease.
However, rapid emergence of resistance to these drugs is
25 well documented, due to the ability of the virus to
rapidly mutate.

DNA-PK is a cellular gene and does not mutate
with the frequency of HIV. Accordingly, inhibitors of
DNA-PK could impede or interrupt HIV replication, and the
30 probability of resistance developing to such inhibitors
would be comparatively low as compared to inhibitors of
viral genes. DNA-PK inhibition is targeted specifically
to the cells desired to be eliminated - newly infected
cells. If DNA-PK is inhibited infected cells will die.
35 However, virus spread will be blocked since the cells

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will die prior to virus production.

Thus, inhibition of DNA-PK is expected to be an extremely useful AIDS therapy. DNA PK_{cs} can be inhibited directly by DNA-PK inhibitors, several of which are currently available (e.g., single stranded DNA, pyrophosphate, 6-dimethyl-aminopurine and the pyridone derivative, OK-1035 (Take et al., Biochem. Biophys. Res. Comm. 215: 41-47, 1995). In addition, as described in the examples, the inventors have shown that Wortmannin inhibits retroviral integration in HeLa cells by inhibiting DNA-PK activity in those cells.

Alternatively, new inhibitors can be developed. DNA-PK_{cs} also can be inhibited indirectly. A recent report indicates that some growth regulators can down-regulate DNA-PK activity (Bandyopadhyay et al., J. Biol. Chem. 273: 1568-1573, 1998). Inhibition of DNA-PK by growth factors may be especially advantageous for anti-HIV therapy due to the low toxicity of growth factors.

The following examples are provided to describe the invention in greater detail. These examples are intended to illustrate and not to limit the invention.

EXAMPLE 1
Lethality of Retroviral Infection to
DNA-PK$_{cs}$ Deficient *Scid* Pre-B Cells

The virus used for the experiments described below is an avian retroviral vector that is replication-competent in avian cells. A schematic diagram of the vector is shown in Figure 1. As indicated in the diagram, the vector includes a murine amphotrophic envelope gene that allows the virus to infect and integrate its DNA in a wide variety of mammalian cells, including mouse and human cells.

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Three independently-derived mouse *scid* pre-B
cell lines (S7, S29 and S33) were used, as well as an
isogeneic normal cell line. The cell lines were infected
with the retroviral vector RCASBP-M (moi approx. 2) and
1:2 and 1:10 dilutions thereof. Controls were (1) mock
infection and (2) infection with heat-inactivated virus).
Cell death was measured at various time points following
infection, using trypan blue staining.

As can be seen from the results shown in Figure
1, cell viability dropped dramatically after infection at
a multiplicity of approximately two infectious units per
cell. No loss of viability was seen upon mock infection
or infection with heat-inactivated virus. The loss of
viability was dependent upon virus concentration. A
reduced effect was observed in cells infected with a 1:2
dilution of the virus, and almost no killing was observed
in cells infected with a 1:10 diluted virus. Viability
of the normal cells was unaffected by infection with the
virus.

These results indicate that infection by a
retrovirus is lethal to *scid* cells.

EXAMPLE 2
Expression of a *neo* Reporter Gene
in Vector-Infected *Scid* Fibroblasts

To verify that the cell killing described in
Example 1 was due to the defect in DNA-PK and not some
other anomaly of transformed *scid* lymphocyte cell lines,
an experiment was performed to determine if the
retroviral vector can become stably integrated in primary
scid fibroblasts.

For these experiments, the avian retroviral
vector described in Example 1 was modified to contain a
neo selectable marker gene, as shown in Figure 2 (RCASPB-
M(*neo*)). Fibroblasts having a stably integrated

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retroviral vector will be resistant to the antibiotic,
G418. Cells (sixth passage) were plated at a density of
1 x 10^5 per 60 mm dish. The following day, cells were
infected with virus. *Neo+* colonies were selected by
5 addition of 500 μg/ml G418, 24 hours later. Results are
shown in Table 1 below.

TABLE 1

Virus Dilution	Normal Fibroblasts (Number of Colonies)		*Scid* Fibroblasts (Number of Colonies)	
10^{-3}	125,	82	9,	7
10^{-4}	26,	34	2,	0
Mock	0,	0	0,	0

        As can be seen in Table 1, no G418-resistant
colonies grew on mock-infected plates. Moreover, the
number of resistant colonies was dependent on virus
concentration. In addition, the number of resistant
25 colonies on plates containing normal fibroblasts was ten
times higher than the number of resistant colonies on
scid fibroblasts.

        The simplest interpretation of these data is
that there were fewer G418 resistant colonies on the *scid*
30 fibroblast plates because, as with the lymphocytes
described in Example 1, most of the infected cells were
killed. Such killing is expected if the partially
integrated viral DNA is recognized as DNA damage by the
cell, but cannot be repaired by the cellular repair
35 mechanism due to the fact that DNA-PK is inactive in
these cells.

EXAMPLE 3
5   **Demonstration that Killing of *Scid* Cells by the**
        Retroviral Vector Requires an Active Integrase

        Two additional derivatives of the avian
retroviral vector described in Example 1 were constructed
10   for the purpose of determining if integration, *per se*, is
the critical factor for the lethality of retroviral
infection observed in *scid* cell lines. These vectors are
shown schematically in Figure 3. In one vector, a DNA
segment was substituted into the integrase gene that
15   included a mutation in the active site of the enzyme.
The resulting conservative substitution in the D, D(35)E
motif inactivates the integrase, but does not affect any
other early events in the virus lifecycle. In a control
vector, the same DNA segment was substituted with a wild-
20   type sequence.

        These retroviral vectors were used to infect
normal and *scid* pre-B cells, as described in Example 1.
Cell viability was measured as described in Example 1.

        Results are shown in Figure 3. The control
25   virus was found to be just as lethal to *scid* cells, but
not control cells, as the parental vector. By contrast,
the integrase-defective virus had no effect on *scid* cell
viability. These results indicate that the lethal event
in retroviral infection of *scid* cells is integration.
30   Normal cells survive because integration can be completed
with the help of the same repair functions that are
critical for DNA strand break repair and V(D)J
recombination, i.e., DNA-PK.

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EXAMPLE 4
Demonstration that Retroviral Infection
Induces the Apoptotic Pathway in *Scid* Cells

5   In *scid* cells, DNA damage is known to induce
apoptosis. This pathway involves a cascade of events
(summarized in Figure 4) that result in visible changes
in the cell. Visual inspection of retrovirus-infected
scid cells suggested that they were dying by apoptosis.
10   To quantify the effect, induction of a characteristic ICE
family protease, caspase 3, was measured in cell lysates.

           Control cells and *scid* cells were infected with
retrovirus in the presence or absence of DEVD-fmk, a
synthetic tetrapeptide that inhibits caspase activity
15   specifically and irreversibly. Normal or *scid* cells were
infected with the virus, lysed 16 hours later, and
caspase activity measured fluorimetrically by formation
of a reaction product of the caspase substrate, DEVD-pNA
(p-nitroanilide). Lysates were normalized by initial
20   cell concentration and protein content.

           Results are shown in Figure 4. A significant
increase in caspase activity (approximately 4X) was
observed in the infected as compared to uninfected *scid*
cells. The specificity of the reaction was verified by
25   addition of the specific inhibitor, which brought the
value down to the level seen in the uninfected cells.
There appeared to be a slight increase in activity in
infected normal cells as well, but this activity was no
greater than the background level in the *scid* cells.
30   These results indicate that retrovirus-infected
scid cells die by apoptosis. Other experimental results
that demonstrate that retrovirus-infected *scid* cells die
by apoptosis are set forth by Daniel et al., Science <u>284</u>:
644-647, 1999 (incorporated by reference herein).

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EXAMPLE 5
Demonstration that DNA-PK Kinase Activity is
Induced by Retroviral Infection of HeLa Cells

5   The ability of retroviral infection to actually
induce DNA-PK kinase activity was determined. Standard
DNA-PK assays cannot detect DNA-PK activity in rodent
cells. However, the activity can be detected in human
(e.g., HeLa) cells because they contain about 50-fold
10   greater amounts of the enzyme than do rodent cells.
Accordingly, human HeLa cells were used for the DNA PK
kinase assay described below.

          A p53 peptide serves as a specific substrate
for phosphorylation by DNA-PK (Bandyopadhyay et al., J.
15   Biol. Chem. 273: 1568-1573, 1998). Using this substrate,
HeLa cells were either treated with exogenous sheared DNA
(known to activate DNA-PK, or they were infected with the
retroviral vector. Controls were (1) cells not exposed
to exogenous sheared DNA and (2) mock-infected cells.

20   Results are shown in Figure 5. The panel on
the left side shows results of the assay for DNA-PK
activity in the presence or absence of sheared salmon
sperm DNA. As can be seen, the kinase was activated in
these control experiments by addition of the exogenous
25   DNA. It is clear that the activity in this assay depends
on all three components of the assay system - p53 peptide
substrate, exogenous sheared DNA, and HeLa cells.

          In the experiments shown in the panel on the
right, nuclear extracts were prepared at various times
30   after infection of HeLa cells with the avian retroviral
vector. In these assays, no exogenous DNA was added to
the assay mixture. As can be seen, DNA-PK activity
increased about 2.5-fold by eight hours after infection.
This corresponds to the time that integration events
35   typically are observed in infected cells. Thus, these
results indicate that DNA-PK is activated in response to
infection with retrovirus.

EXAMPLE 6
Demonstration that *Scid* Cells are Killed by
Infection with an HIV-Based Retroviral Vector

The experiments described in the foregoing examples were performed using an avian retroviral vector. To determine if the observed responses can be generalized to other retroviruses, *scid* cells were infected with a murine leukemia virus and with an HIV-based vector that was pseudotyped with the VSV G protein to allow it to enter rodent cells.

Figure 6 shows the results of the experiments with the HIV-based vector, which were similar to the results observed with the murine virus. As can be seen, cells infected with the HIV vector, at a multiplicity of about one infectious unit per cell, showed a loss of viability over time, while mock-infected cells or cells infected with heat-inactivated vector did not. Moreover, normal cells were not affected by any treatment.

The HIV vector used included a β-galactosidase reporter gene that must be integrated into the chromosome to be expressed. As shown in Figure 6, measurement of β-galactosidase activity in lysates of infected cells shows that the *scid* lines had only 30-50% of the activity of the normal cell line. Taken together, these results indicate that lethality to DNA-PK deficient *scid* cells after retroviral infection (at the integration step) is a generalized effect of retroviruses.

EXAMPLE 7
Effects of Wortmannin

Retroviral integration in HeLa cells. The effect of Wortmannin on retroviral integration in HeLa cells (G418 resistant colonies) was investigated. HeLa cells were plated at 10^5 cells per 60 mm dish and infected

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with the IN+ virus that carries a *neo* marker, which was
described in Example 2. *Neo*+ colonies were selected by
addition of 1 mg/ml G418, 24 hours later.

Results are shown in Figure 8. As can be seen,
5   Wortmannin inhibits retroviral integration in HeLa cells
in a concentration-dependent manner. Integration is
essentially abrogated with 20 μM Wortmannin.

Growth of HeLa cells. HeLa cells were plated
at a concentration of 10^5 cells per 60 mm dish. Cells
10 were exposed to various concentrations of Wortmannin and
counted three days after exposure. Results are shown in
Figure 9. As can be seen, Wortmannin had no effect on
the growth of HeLa cells in the 1-20 μM range, even
though Wortmannin inhibits retroviral integration in the
15 cells at that concentration range (Figure 8).

DNA-PK-related activity. HeLa cells were
treated overnight with Wortmannin. After that, cells
were lysed and a DNA-PK activity assay was performed as
described in earlier examples (see also Daniel et al.,
20 1999, *supra*. Results are shown in Figure 10. As can ben
seen, Wortmannin inhibits DNA-PK activity in HeLa cells.
Inhibition of the DNA-PK-related kinase activity
correlates with inhibition of retroviral integration.

Viability of S33 cells. DNA-PK kinase activity
25 is the primary target of the DNA-PK inhibitor Wortmannin
in vivo. However, Wortmannin also inhibits other DNA-PK
related kinases, such as ATM, though at high
concentrations.

A percentage of *scid* cells survive infection
30 (Example 1 and Daniel et al., 1999, *supra*). To determine
if this "leakiness" is due to a DNA-PK-related kinase,
scid cells were treated with Wortmannin and infected with
virus (RCASB-M; see Figure 2). Results are shown in
Figure 11. As can be seen, at 2 μM, Wortmannin enhances
35 the lethal-effect of the virus. This finding suggests

that a DNA-PK-like kinase is responsible for the
leakiness of the virus-mediated *scid* cell killing.

             **Inhibition of G418 colony formation in ATM cell
lines.** About 10% of the DNA-PK-deficient *scid* cells are

5   susceptible to retroviral infection and integration.
Results shown in Figure 11 suggest that this leakiness is
due to an alternative pathway that is mediated by a DNA-
PK-like kinase. The ATM kinase is closely related to
DNA-PK and also plays a role in DNA repair. It was

10   therefore investigated whether ATM could be involved in
an alternative pathway of retroviral integration.

            Retrovirus (IN+ - see Figure 3) can still
integrate into MO59J cells, which have no DNA-PK (see
Table 2 below). These data confirm that the above

15   mentioned "leakiness" is not due to a residual DNA-PK
activity. Treatment of MO59J cells (DNA-PK null) with
Wortmannin confirms the alternative pathway is
Wortmannin-sensitive and thus involved DNA-PK-related
kinase, but not DNA-PK. This finding is consistent with

20   the results presented in Figure 11.

            The primary target of Wortmannin is DNA-PK
kinase. The ATM kinase is inhibited at higher Wortmannin
concentrations than those required to inhibit the DNA-PK
kinase. Cells deficient in the ATM kinase were infected

25   with the IN+ virus carrying a *neo* marker, then treated
with Wortmannin. Results are shown in Table 2.

TABLE 2

cells	HeLa	AT2SF	MO59J	HeLa	AT5B1	CHO-K1	XR-1
DNA-PK	+	+	−	+	+	+	-(XRCC4-)
ATM	+	−	+	+	−	+	+

Wort. (μM)	percentage of G418^r colonies						
0	100	100	100	100	100	100	100
1	56	11	43	65	4	77	66
5	45	5	35	24	1	35	44

As shown, treatment with Wortmannin led to a dramatic
reduction in retroviral integration in ATM cells, much
faster than in HeLa cells (Table 2 shows two different
experiments with HeLa cells and two ATM-deficient lines,

5   AT2SF and AT5B1). It is concluded that ATM is involved
in the alternative pathway of retroviral integration.
Moreover, as can be seen from the Table, G418 colony
formation in XRCC4- cells was still sensitive to
Wortmannin. Thus, the XRCC4 gene, which is a part of the

10   DNA-PK pathway, appears not to be involved in the
alternative pathway.

        The present invention is not limited to the
embodiments described and exemplified above, but is

15   capable of variation and modification without departure
from the scope of the appended claims.

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What is claimed:

1. A method of identifying cellular enzymes needed to complete integration of retroviral DNA into a genome, which comprises:

a) providing a pair of retroviral vectors, one of the pair encoding a functional integrase and the other of the pair encoding a non-functional integrase;

b) providing samples of a cell line suspected of lacking at least one enzyme needed to complete integration of the retroviral vector into the genome of the cell line;

c) infecting one of the samples with the retroviral vector encoding the functional integrase and another of the samples with the retroviral vector encoding the non-functional integrase; and

d) measuring cell death in each of the samples, a greater amount of cell death in the sample infected with the retroviral vector encoding the functional integrase, as compared with the sample infected with the retroviral vector encoding the non-functional integrase, being indicative that the enzyme lacking in the cell line is needed to complete integration of the retroviral DNA into the cell's genome.

2. The method of claim 1, wherein the cell line is a mouse cell line.

3. The method of claim 1, wherein the pair of retroviral vectors are derived from a retrovirus selected from the group consisting of ASLV, MoMLV and HIV.

4. The method of claim 3, wherein the pair of retroviral vectors are RCA8KSneo and RCAD64Eneo.

5.   The method of claim 1, which further
comprises providing samples of a control cell line
capable of completing the integration of the retroviral
DNA.

6.   The method of claim 1, wherein the cell
death is measured by determining the percentage of viable
cells remaining in the samples after the infecting.

7.   The method of claim 1, wherein the cell
death is measured by measuring a cellular component of
the apoptosis pathway.

8.   The method of claim 7, wherein the cellular
component comprises a caspase.

9.   The method of claim 1, wherein the pair of
retroviral vectors further comprises a selectable marker
gene that confers resistance only if the retroviral
vector is stably integrated into the cell's genome, and
the cell death is measured by plating the infected cells
on selection medium and thereafter counting colonies on
the medium.

10.   The method of claim 1, wherein the pair of
retroviral vectors further comprises a reporter gene that
is expressed only if the retroviral vector is stably
integrated into the cell's genome, and the cell death is
measured by detecting the product of the reporter gene.

11.   A test kit for identifying cellular
enzymes needed to complete integration of retroviral DNA
into a genome, which comprises a container containing:
        a) a pair of retroviral vectors, one of
the pair encoding a functional integrase and the other of

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the pair encoding a non-functional integrase; and

         b) instructions for using the pair of
retroviral vectors to identify the cellular enzymes.

5   12.   The test kit of claim 11, which further
comprises a control cell line capable of completing the
integration of the retroviral DNA.

         13.   The test kit of claim 11, which further
10   comprises reagents for measuring the cell death.

         14.   A method of identifying an agent that
interferes with a retroviral life cycle at a step
preceding completion of integration of retroviral DNA
15   into a host cell genome, which comprises:

         a) providing a cell line that is unable to
perform a DNA repair step in integration of retroviral
DNA into the cell's genome;

         b) infecting with a retrovirus one sample
20   of the cell line in the presence of a test compound
suspected of interfering with the retroviral life cycle;

         c) infecting with a retrovirus an
equivalent sample of the cell line in the absence of the
test compound; and

25   d) measuring cell death of the infected
samples of cell lines, a decrease in amount of cell death
in the presence of the test compound as compared with in
the absence of the test compound being indicative that
the test compound is an agent that interferes with the
30   retroviral life cycle at a step preceding completion of
retroviral DNA integration into the cell's genome.

         15.   The method of claim 14, wherein the cell
line comprises a non-functional DNA-dependent protein
35   kinase.

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16. The method of claim 15, wherein the cell
line is derived from a *scid* mouse.

17. The method of claim 14, wherein the
retrovirus is selected from the group consisting of ASLV,
MoMLV and HIV.

18. The method of claim 14, which further
comprises infecting in the presence and absence of the
compound a control cell line capable of completing the
integration of the retroviral DNA.

19. The method of claim 14, which further
comprises infecting additional samples of the cell line
with a retrovirus which produces a non-functional
integrase.

20. The method of claim 14, wherein the cell
death is measured by determining the percentage of viable
cells remaining in the samples after the infecting.

21. The method of claim 14, wherein the cell
death is measured by measuring a cellular component of
the apoptosis pathway.

22. The method of claim 21, wherein the
cellular component comprises a caspase.

23. The method of claim 14, wherein the genome
of the retrovirus further comprises a selectable marker
gene that confers resistance only if the retroviral DNA
is stably integrated into the cell's genome, and the cell
death is measured by plating the infected cells on
selection medium and thereafter counting colonies on the
medium.

24. The method of claim 14, wherein the genome
of the retrovirus further comprises a reporter gene that
is expressed only if the retroviral DNA is stably
integrated into the cell's genome, and the cell death is
5   measured by detecting the product of the reporter gene.

25. A test kit for identifying an agent that
interferes with a retroviral life cycle at a step
preceding completion of integration of retroviral DNA
10   into a host cell genome, which comprises:
              a) a cell line that is unable to perform a
DNA repair step in integration of retroviral DNA into the
cell's genome;
              b) a retroviral vector; and
15   c) instructions for using the cell line
and the vector to identify agents that interfere with the
retroviral life cycle.

26. The test kit of claim 25, wherein the cell
20   line is derived from a *scid* mouse.

27. The test kit of claim 25, which further
comprises a control cell line capable of completing the
integration of the retroviral DNA.
25

28. The test kit of claim 25, which further
comprises a control retroviral vector that encodes a non-
functional integrase.

30   29. The test kit of claim 25, which further
comprises reagents for measuring the cell death.

30. A method of interfering with retroviral
replication in a host cell infected with the virus, which
35   comprises inhibiting an enzyme of the host cell needed to

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perform the repair step of retroviral DNA integration
into the host cell genome.

5   31. The method of claim 30, wherein the enzyme
is a DNA-dependent protein kinase.

     32. The method of claim 30, wherein the enzyme
is an Ataxia telangiectasia mutated kinase.

10   33. The method of claim 30, wherein the host
cell is a human cell.

     34. The method of claim 30, wherein the
inhibiting is accomplished by exposing the cell to an
15 exogenous agent that inhibits the enzyme.

*Viability is determined by trypan blue staining
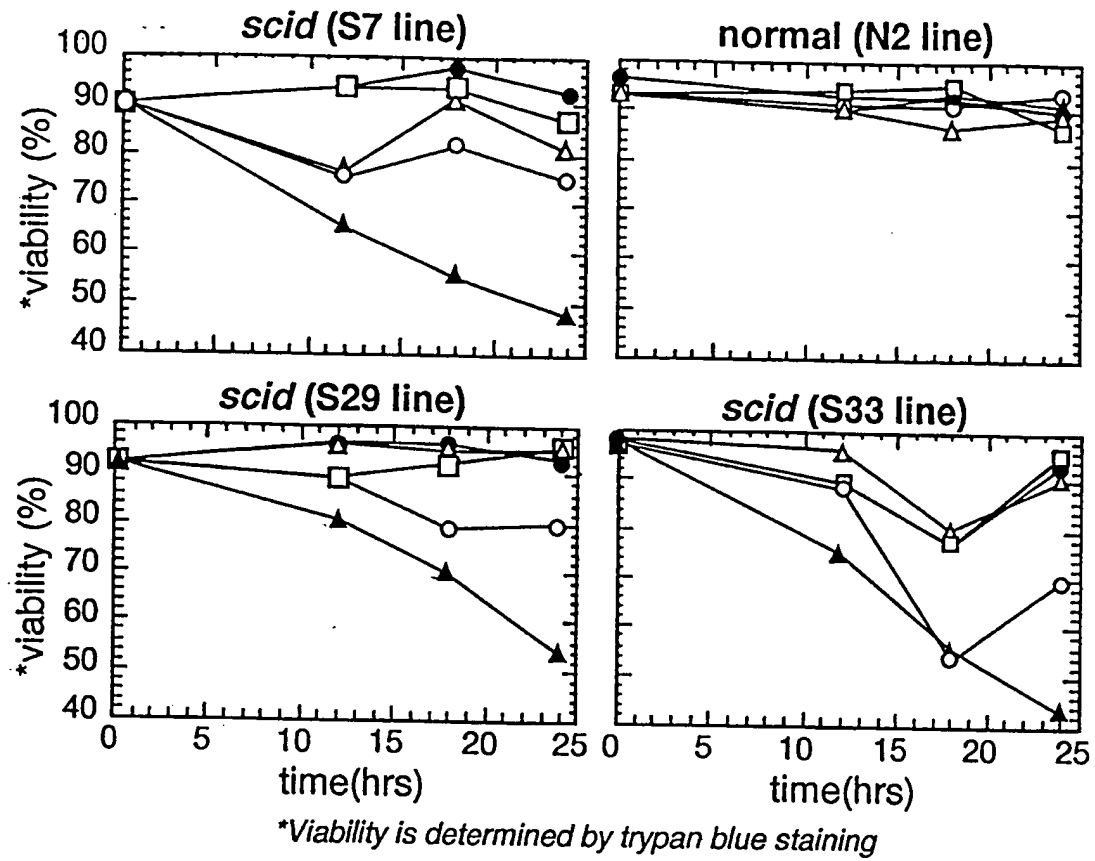
—▲— RCASBP-M (moi≈2)   —●— mock infection
—○— RCASBP-M (1:2 dilution)   —□— heat-inactivated RCASBP-M
—△— RCASBP-M (1:10 dilution)
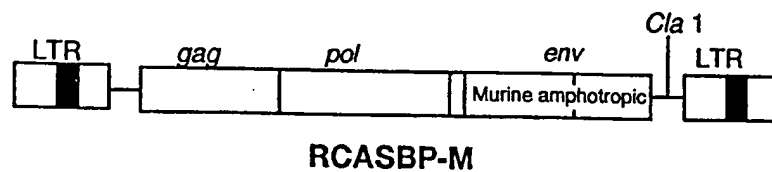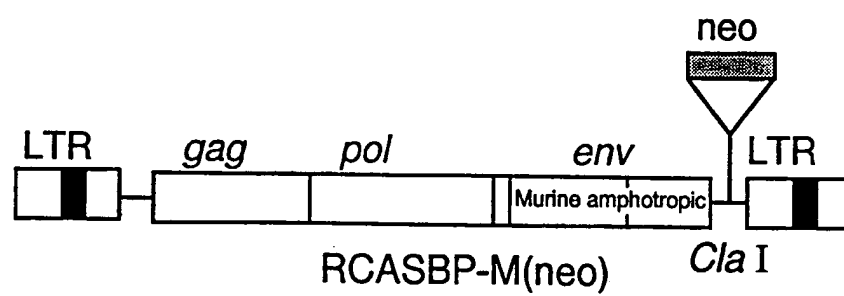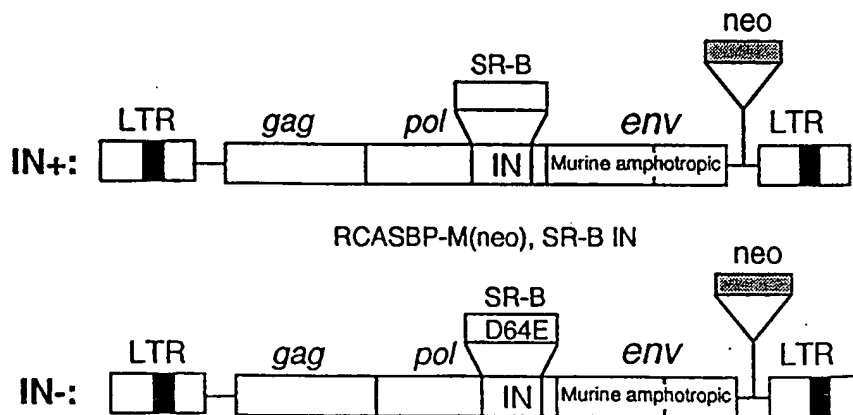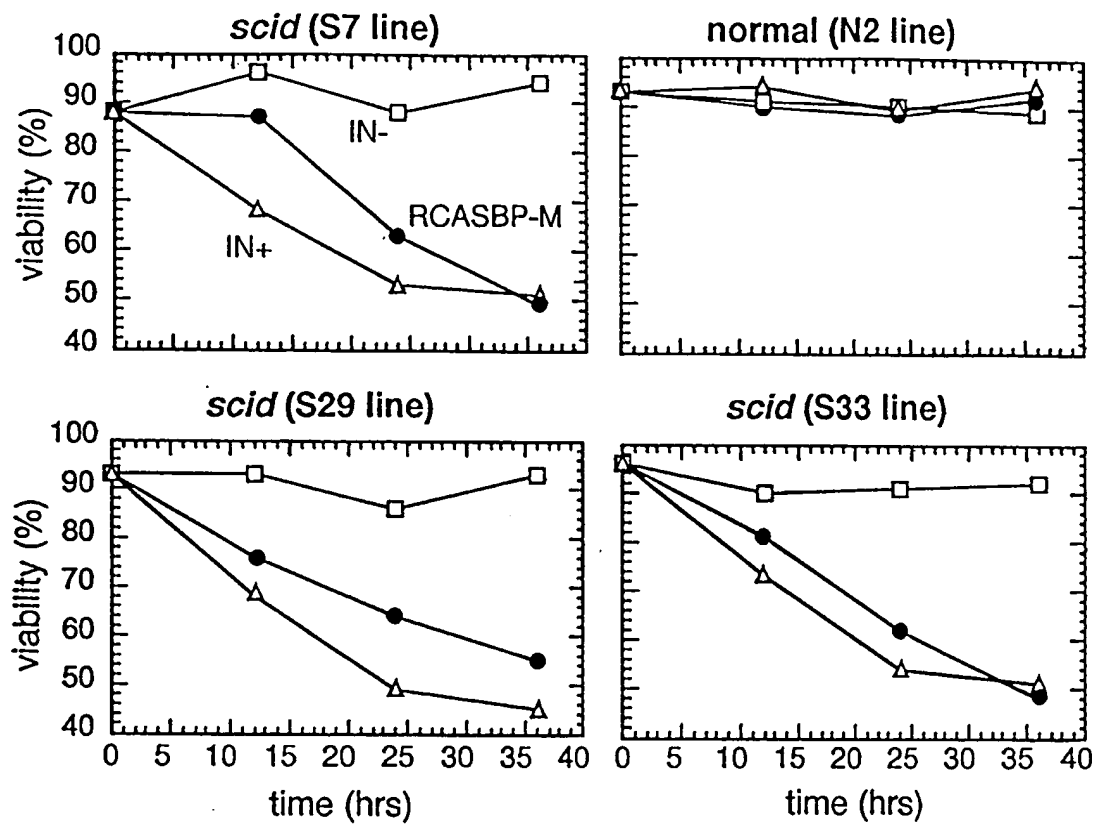
Figure 1

Figure 2

3/11

Figure 3

4/11
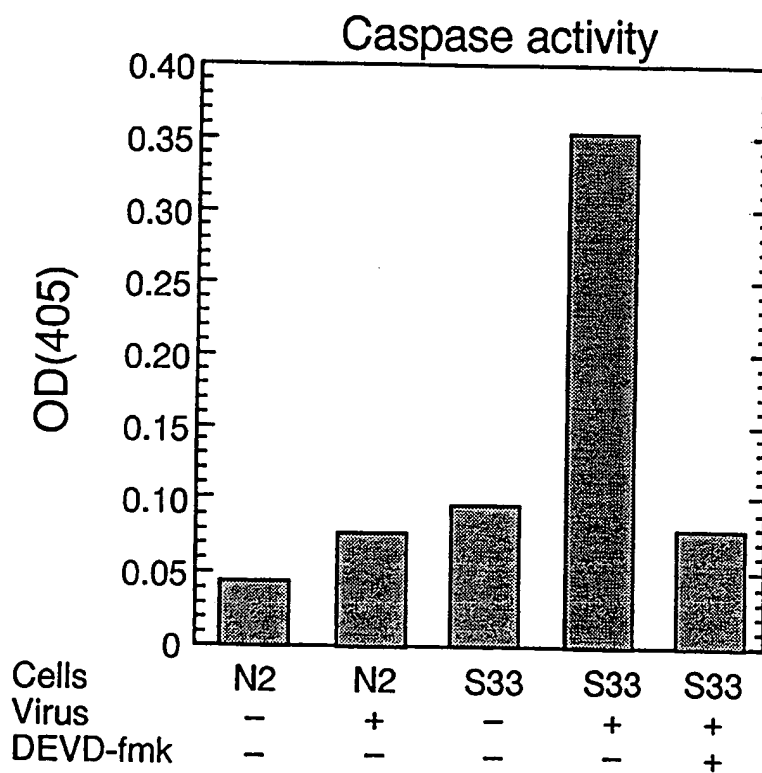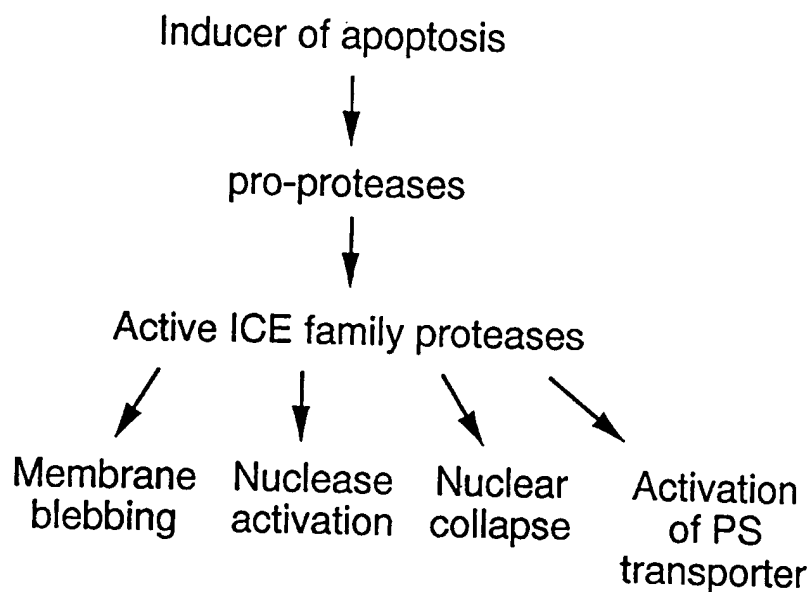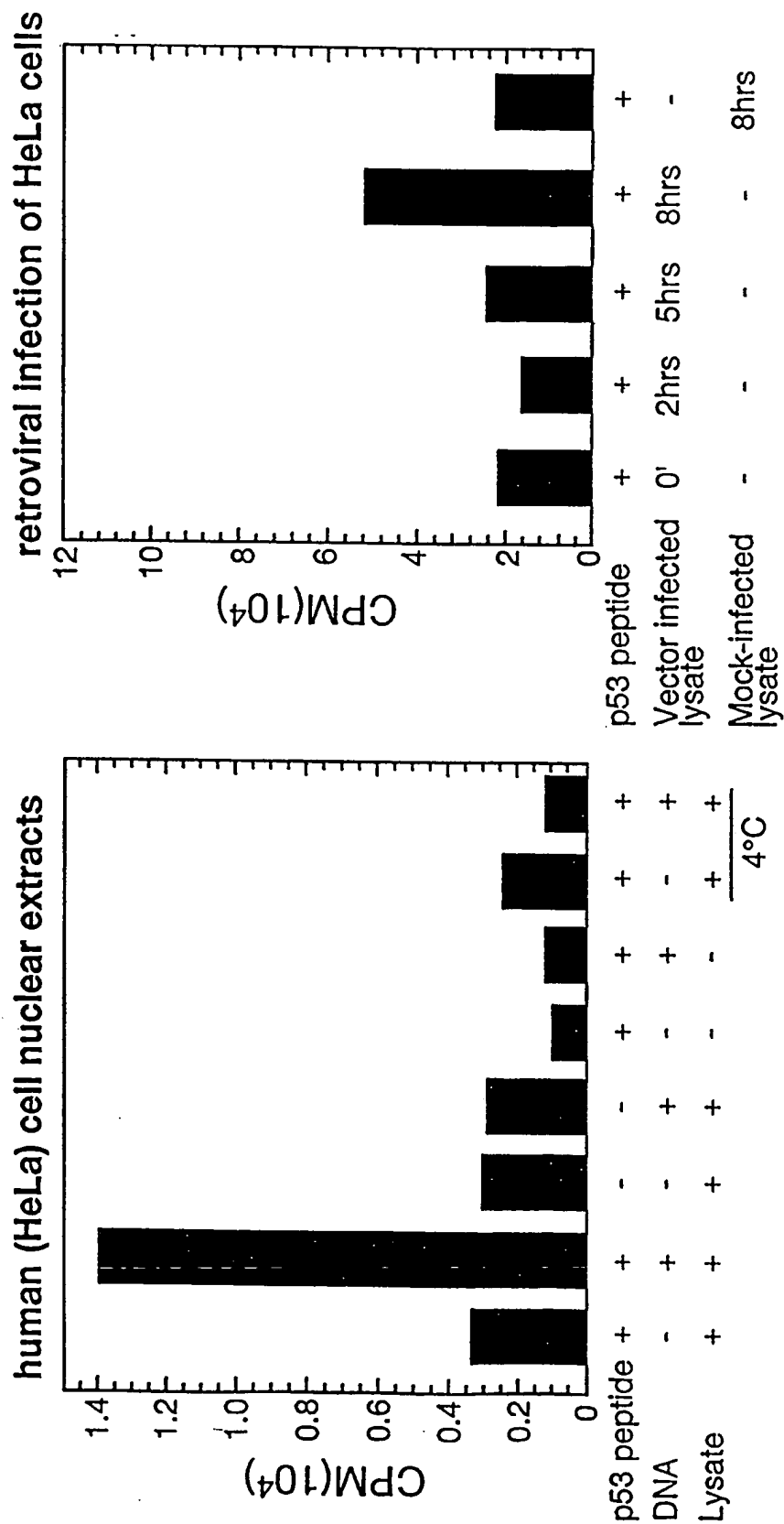
Inducer of apoptosis

↓

pro-proteases

↓

Active ICE family proteases

↙   ↓   ↓   ↘

Membrane   Nuclease   Nuclear   Activation
blebbing   activation collapse   of PS
                                   transporter
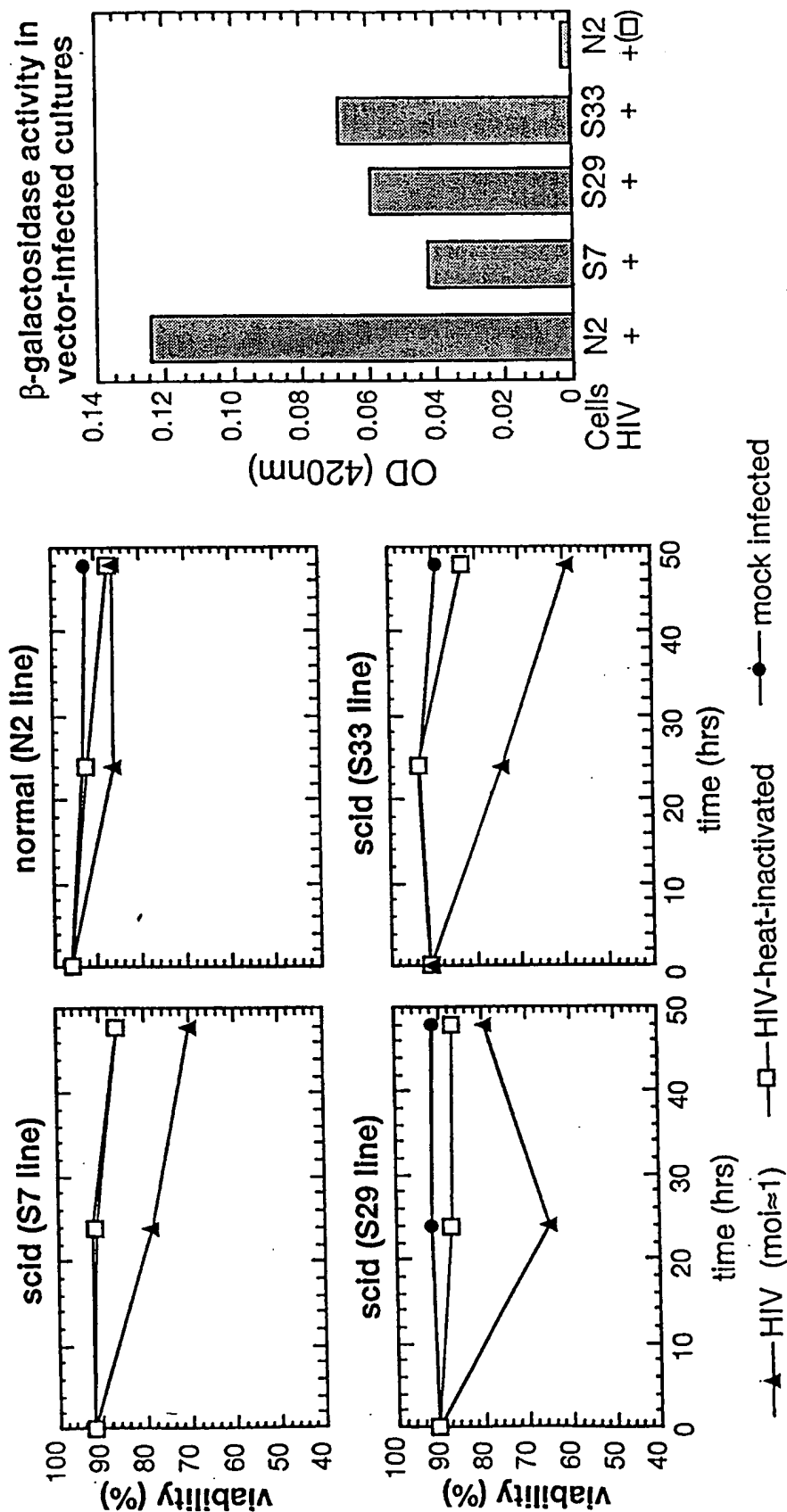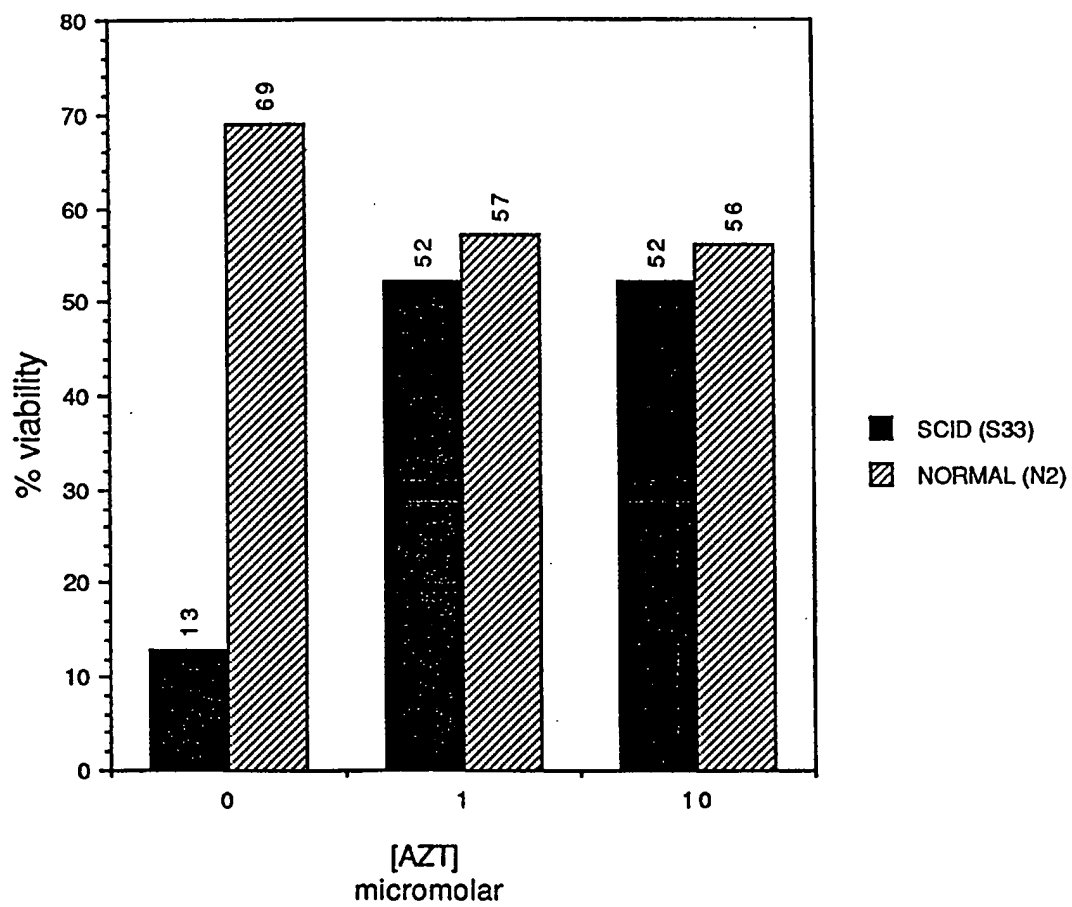
Figure 4
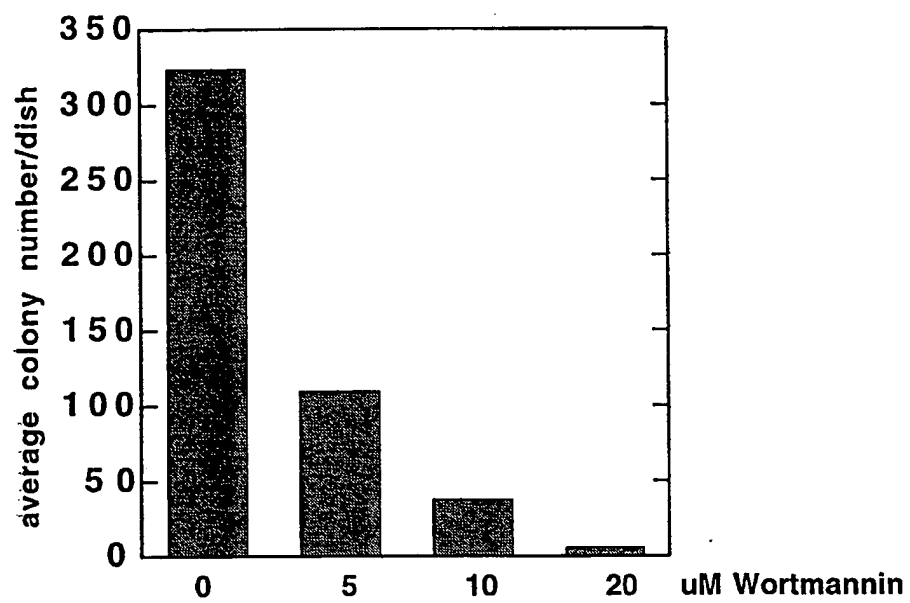
Figure 5

Figure 6

Figure 7
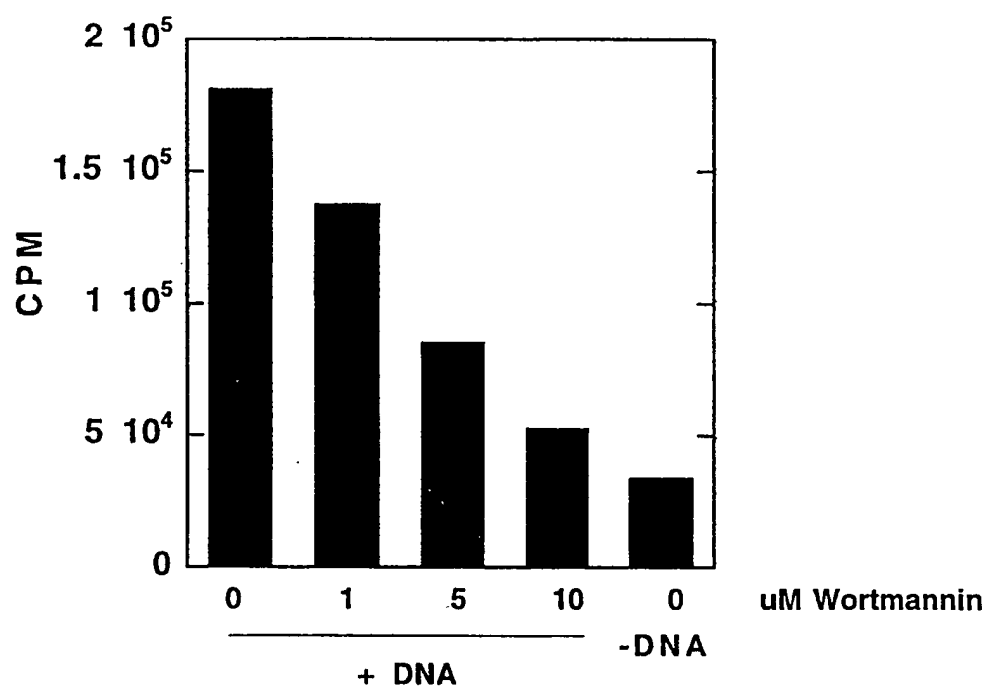
Figure 8
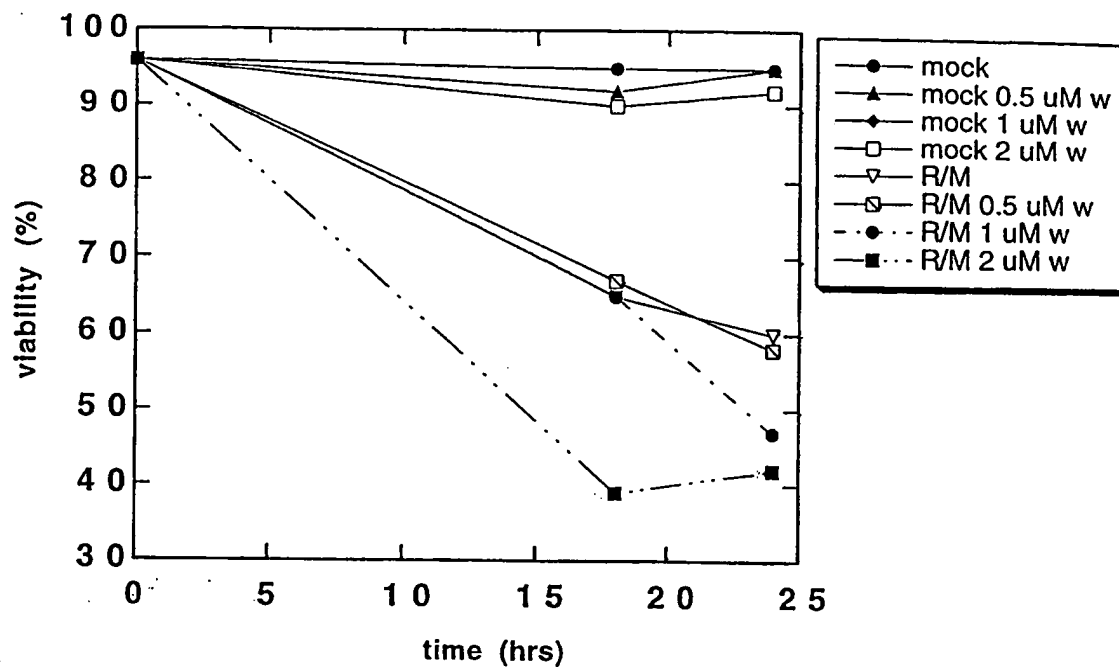
Figure 9

Figure 10

Figure 11

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER	

IPC(6)   :C12Q 1/00, 1/68, 1/70; C12N 13/00, 9/00; GO1N 33/573
US CL   :435/4, 5, 6, 7.4, 173.3, 183
According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

 U.S. :   435/4, 5, 6, 7.4, 173.3, 183

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

 WEST, BIOSIS, USPATFULL

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	US 5,955,644 A (HASTY et al) 21 September 1999, see entire document.	1-34N
A,P	DANIEL, R., et al. A Role for DNA-PK in Retroviral DNA Integration, Science, 23 April 1999. Vol. 284, No. 5414, pages 644-647, see entire document.	1-34

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
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"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 FEBRUARY 2000	0 2 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer LAURIE SCHEINER
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